PATENT
5842-00503

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08/08/2006 /Pamela Gerik/
Date Pamela Gerik

DECLARATION UNDER 37 C.F.R. § 1.131

I, C. Steven McDaniel, hereby declare and state that:

1. I am a named inventor in the above-identified patent application, which is U.S. Patent Application No. 08/252,384 filed on June 1, 1994, which is a continuation of U.S. Serial Number 07/928,540 filed August 13, 1992, now abandoned, which is a divisional application of U.S. Serial Number 07/344,258 filed April 27, 1989, now abandoned.

2. I have been informed that in the present application certain claims have been rejected in reference to U.S. Patent No. 5,484,728 to Serdar et al., which was issued on January 16, 1996 and was filed on November 1, 1994. U.S. Patent No. 5,484,728 to Serdar et al. is a continuation of U.S. Serial Number 898,973 filed June 15, 1992, now abandoned, which is a division of U.S. Serial Number 312,503 filed February 17, 1989, which is a continuation-in-part of U.S. Serial Number 237,255 filed August 26, 1988, now abandoned.

CONCEPTION

3. As supported below, I, along with Frank M. Raushel and James R. Wild, conceived of the subject matter claimed in the present application within the United States before August 26, 1988. The subject matter includes an isolated nucleic acid molecule including a nucleotide sequence encoded in an organophosphorous acid anhydrase.

4. Exhibit A attached hereto is a true copy of a paper entitled "Cloning and Sequencing of a Plasmid-Borne Gene (opd) Encoding a Phosphotriesterase" which was submitted to, accepted by, and published by the Journal of Bacteriology all before August 26, 1988.

5. Fig. 3 of Exhibit A illustrates a nucleotide sequence of the *opd* gene of the presently claimed case. In particular, Fig. 3 of Exhibit A illustrates the nucleotide sequence of an *opd* gene depicted in Fig. 1 and referenced in pending claim 73 of the presently claimed case. The first full paragraph under the heading of "Discussion" on page 2309 of Exhibit A specifies the *opd* gene is encoded in organophosphorous acid anhydrases.

REDUCTION TO PRACTICE AND DILIGENCE

6. From at least a time just prior to August 26, 1988 through the filing of parent U.S. Patent Application No. 07/344,258 filed on April 27, 1989, plans were undertaken to prepare the captioned patent application. I did not abandon, suppress, or conceal the ideas set forth in the claimed invention during at least the time beginning just prior to August 26, 1988 through the filing of the parent application on April 27, 1989.

7. Upon information and belief, it is my informed understanding that diligence in reducing the invention to practice was therefore maintained from at least as early as just prior to August 26, 1988 through the filing of the parent application on April 27, 1989.

8. I hereby declare that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

/C. Steven McDaniel/
C. Steven McDaniel

Date: August 8, 2006

EXHIBIT A

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Cloning and Sequencing of a Plasmid-Borne Gene (*opd*) Encoding a Phosphotriesterase

C. STEVEN MCDANIEL, LINDA L. HARPER, AND JAMES R. WILD*

Department of Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843

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Plasmid pCMS1 was isolated from *Pseudomonas diminuta* MG, a strain which constitutively hydrolyzes a broad spectrum of organophosphorus compounds. The native plasmid was restricted with *Pst*I, and individual DNA fragments were subcloned into pBR322. A recombinant plasmid transformed into *Escherichia coli* possessed weak hydrolytic activity, and Southern blotting with the native plasmid DNA verified that the DNA sequence originated from pCMS1. When the cloned 1.3-kilobase fragment was placed behind the *lacZ* promoter of M13mp10 and retransformed into *E. coli*, clear-plaque isolates with correctly sized inserts exhibited isopropyl-β-D-thiogalactopyranoside-inducible whole-cell activity. Sequence determination of the M13 constructions identified an open reading frame of 975 bases preceded by a putative ribosome-binding site appropriately positioned upstream of the first ATG codon in the open reading frame. An intragenic fusion of the *opd* gene with the *lacZ* gene produced a hybrid polypeptide which was purified by β-galactosidase immunoaffinity chromatography and used to confirm the open reading frame of *opd*. The gene product, an organophosphorus phosphotriesterase, would have a molecular weight of 35,418 if the presumed start site is correct. Eighty to ninety percent of the enzymatic activity was associated with the pseudomonad membrane fractions. When dissociated by treatment with 0.1% Triton and 1 M NaCl, the enzymatic activity was associated with a molecular weight of approximately 65,000, suggesting that the active enzyme was dimeric.

Synthetic organophosphorus neurotoxins are used extensively as agricultural and domestic pesticides including insecticides, fungicides, and herbicides. Naturally occurring bacterial isolates capable of metabolizing this class of compounds have received considerable attention (20, 25) since they provide the possibility of both environmental and in situ detoxification (reviewed in reference 18). *Pseudomonas putida* MG and *Flavobacterium* spp. have been shown to possess the ability to degrade an extremely broad spectrum of organophosphorus phosphotriesters as well as thiol esters (4, 6). Recently, certain mammalian neurotoxins, such as diisopropyl phosphonofluoridate (1) and Soman (1,2,2-trimethylpropyl-methylphosphonofluoridate; J. DeFrank, personal communication), have been shown to be hydrolyzed by selected bacteria. Several of the bacterial strains possess constitutively expressed phosphotriesterases with broad substrate ranges including many commonly used organophosphorus pesticides (4, 6). None of these strains has shown the ability to utilize these neurotoxins as sole nutrient or energy sources, thus making mutant selection difficult (C. S. McDaniel and J. R. Wild, Arch. Environ. Contam. Toxicol., in press). The hydrolysis of organophosphorus compounds by the pseudomonad phosphotriesterase has been shown to proceed via nucleophilic addition of a molecule of water across the acid anhydride bond (V. E. Lewis, W. J. Donarski, J. R. Wild, and F. M. Raushel, *Biochemistry*, in press). (The class of enzymes EC 3.1.3 [which includes diisopropyl phosphorofluoridase and somanase] to which the *opd* gene product belongs was recently renamed "organophosphorus acid anhydrase" at the 1987 DFPase Workshop held at Woods Hole Marine Biological Laboratories, Woods Hole, Mass. Synonyms which have been used include phosphotriesterase, parathion hydrolase, paraoxonase, and parathion aryl esterase.) In addition, applications of enzymatic hydrolysis have been limited due to lack of economical fermentations of the native soil bacteria (19).

Two bacterial strains from the closely related genera *Pseudomonas* and *Flavobacterium* encode organophosphorus-degrading genes (*opd*) on large plasmids (40 to 65 kilobases [kb]) (15, 23, 24), while the locations of the degradative genes are unknown in other bacteria (13, 22). In the present study, the *opd* gene from *Pseudomonas diminuta* was sequenced and its membrane-associated gene product was expressed in heterologous genetic backgrounds from several promoter systems. The native enzyme has been partially purified, allowing molecular weight estimation, and the open reading frame has been verified by direct amino acid sequence of a purified β-galactosidase fusion polypeptide.

MATERIALS AND METHODS

Bacterial strains and plasmids. *P. diminuta* MG is the original host of pCMS1 and was obtained from the laboratory of D. Gibson, *Escherichia coli* strains HB101-4442 (auxotrophic for uracil and proline; 10) and JM103 were used as host cells for the cloning vectors, pBR322 (3) and phage M13mp10 (14), respectively. The recombinant plasmid pBR322-038 contained a 1.3-kb *Pst*I fragment of pCMS1 cloned into the ampicillin resistance gene of pBR322. M13mp-038/008 and M13mp10-038/004 were oppositely oriented phage constructions which were enzymatically active or inactive, depending upon the orientation of the 1.3-kb fragment of pBR322-038. Hybrid gene fusions were produced in plasmid pMC1403 and expressed in *E. coli* CQ4 (28).

Media and growth conditions. Cultures were grown at 32°C (*P. diminuta*) or 37°C (*E. coli*). Nutrient medium consisted of 10 g of tryptone (Difco Laboratories), 5 g of yeast extract (Difco), and 5 g of NaCl per liter (TYE). TF minimal medium (17) was used for *E. coli* strains and was supplemented with uracil (50 μg/ml), proline (25 μg/ml), vitamin B_1 (0.01%),

* Corresponding author.

Casamino Acids (0.1%), glucose (0.2%), and antibiotics (25 to 50 μg/ml) as required.

Isolation of plasmid DNA. Standard protocols for the isolation of DNA from *E. coli* for plasmid (7) or phage (14) have been previously described. Isolation of predominantly covalently closed circular plasmid DNA from *P. diminuta* was accomplished via a mild lysis procedure modified from that of Berns and Thomas (2).

Cloning and sequencing of *opd* from the native plasmid. The *Pst*I restriction fragments of pCMS1 were inserted into pBR322, inactivating the ampicillin gene (Focus 5:3, Bethesda Research Laboratories [BRL], Gaithersburg, Md., 1983). The resulting recombinant plasmids were used to transform competent HB101-4442, and tetracycline-resistant (Tc^r) colonies were selected and evaluated for ampicillin sensitivity (Ap^s). The plasmid structure of selected Tc^r Ap^s transformants was determined, and clones representing the different inserts were analyzed for activity.

The 1.3-kb *Pst*I insert of pBR322-038 was excised from its vector, purified by preparative agarose gel electrophoresis using a modified freeze-squeeze phenol procedure (S. A. Benson, Biotechniques **March/April:66–67**, 1984), and subsequently introduced into the multiple cloning site of M13mp10. The resulting recombinant molecules were transformed into competent *E. coli* JM103 cells, and clear-plaque isolates were selected. All subsequent manipulations of viral recombinant DNAs were performed according to the methodology of the BRL "M13 Cloning/Dideoxy Sequencing Manual." A variety of 5′ and 3′ deletions of pBR322-038 were constructed, using various restriction sites surrounding the *opd* gene (*Bam*HI, *Ava*I, *Nru*I, *Sal*I, *Sph*I). In addition, 3′ exonuclease III deletions were utilized to identify gene boundaries.

Dideoxy sequencing was accomplished by the method of Sanger as detailed in the BRL "M13 Cloning/Dideoxy Sequencing Manual." In cases where GC compaction was evident, reverse transcriptase as well as the Klenow fragment of DNA polymerase was used (BRL, manufacturer's protocols). Oligonucleotide primers were synthesized using phosphoramidite chemistry with an Applied Biosystems Synthesizer according to the manufacturer's recommendations.

The 5′ region of the *opd* gene was subcloned into the β-galactosidase gene for the purposes of producing a *lacZ* fusion polypeptide. The 1.3-kb *opd* fragment was restricted with *Ava*I (see Fig. 3); the staggered restriction fragment was end-filled with DNA polymerase (Klenow fragment) and blunt-end ligated into the 5′ *Sma*I cloning site of the *lacZ* fragment of pMC1403 (28). This hybrid genetic construction was transformed into *E. coli* CQ4 (5).

Production of *opd* probes and Southern DNA hybridization. Various constructions containing the *opd* gene sequence (pCMS1, pBR322-038, M13mp10-038/008, and the inactive M13mp10-038/004) were evaluated for hybridization with the *opd*-containing fragment. Undigested controls and corresponding *Pst*I-digested samples were electrophoresed on a 0.7% agarose-TBE gel (89 mM Tris base, 89 mM borate, and 2.5 mM sodium EDTA). After photography, the gels were transferred (26) onto nitrocellulose paper and probed with ^{32}P-labeled nick-translated pBR322-038 DNA.

Phosphotriesterase assay. Routine analysis of parathion hydrolysis in whole cells was accomplished by suspending cultures in 10 mM Tris hydrochloride (pH 8.0) containing 1.0 mM sodium EDTA (TE buffer). Cell-free lysates were assayed using sonicated extracts as described previously (10) in 0.5 ml of TE buffer. The suspended cells or cell extracts were incubated with 10 μl of substrate (100 μg of parathion in 10% methanol), and *p*-nitrophenol production was monitored at a wavelength of 400 nm. To induce the gene under *lac* control, 1.0 μmol of isopropyl-β-D-thiogalactopyranoside (Sigma) per ml was added to the culture media.

Column chromatography, affinity chromatography, and protein sequencing. *P. diminuta* cells from a 200-liter fermentation (grown in the National Institutes of Health-Department of Energy-sponsored fermentation facility of the Department of Biochemistry and Biophysics, Texas A&M University) were harvested by a continuous-flow centrifuge and suspended in 2.0 liters of 1.0 M NaCl. Samples of this suspension were agitated in a Waring blender for 30 s, and the resulting suspension was centrifuged at 400 × *g* for 10 min. Portions of this suspension (5.0 ml) were sonicated, treated with 0.1% Triton X-100, and stirred at room temperature for 2 h before chromatography.

The molecular weight of the native enzyme was determined by ascending Sephadex G-200 chromatography in the presence of 50 mM CHES buffer [2-(N-cyclohexyl-amino)ethanesulfonic acid (pH 9.0)] at 4°C. Enzymatic activity was located by introducing 50-μl aliquots of column fractions (2.0 ml) into a reaction volume of 0.8 ml containing 0.2 mM paraoxon and 50 mM CHES buffer (pH 9.0).

Purification of hybrid β-galactosidase proteins encoding the 5′ region of the *opd* gene was achieved by immunoaffinity chromatography (28) and preparative gel electrophoresis. Gas-phase sequencing of the purified fusion polypeptide (Applied Biosystems 470A Sequencer, Applied Biosystems 120A On-line-PTH Analyzer, TAES Biotechnology Support Laboratory) was accomplished by the methods of Hewick et al. (12).

RESULTS

Partial purification and molecular weight estimation. Upon cellular disruption of the native *P. diminuta* strain by sonication or French pressure cell disruption, 80 to 90% of the activity was associated with the particulate fraction. It was possible to release activity from the particulate complex by treatment with 0.1% Triton X-100 or 0.2% Tween 20 without significant loss of activity. When these enzyme preparations were analyzed by Sephadex G-200 column chromatography, the molecular weight of the enzymatically active fractions was 60,000 to 65,000.

Cloning of pCMS1 into pBR322. The entire DNA from the degradative plasmid was digested with *Pst*I (generating fragments of approximately 18.5, 17.3, 5.3, 4.3, 1.7, 1.6, 1.3, and 0.8 kb) and was subcloned into pBR322 within that vector's ampicillin gene. Cell-free lysates of Ap^s clones selected from the Tc^r transformants of *E. coli* HB101-4442 were tested for activity. One single-colony isolate was selected for its ability to hydrolyze parathion, and the expected phenotype (Tc^r Ap^s; auxotrophy for uracil and proline; parathion hydrolysis) was verified. A 5.6-kb, CsCl-purified plasmid was isolated from this strain and used to transform competent HB101-4442 cells, regenerating the phenotype and demonstrating that the hydrolytic activity was mediated by the recombinant plasmid. Other isolates with a similarly sized insert but lacking the hydrolytic activity were subsequently shown to have an orientation opposite to that of the active clone (data not shown). This observation demonstrated that the orientation of the *opd*-containing fragment within the pBR322 vector was critical to heterologous expression. Thus, it appeared that the expression of the 1.3-kb fragment (approximately 1 to 2% of the

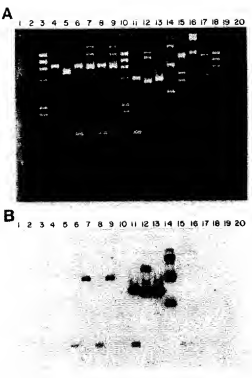

FIG. 1. Southern hybridization of *opd* probe (pBR322-038; pBR322 plus the 1.3-kb *opd* fragment) with the native plasmid and subclones. (A) Agarose gel electrophoresis of *opd*-containing DNAs. Lanes 6, 8, 11, and 15 contain *Pst*I-digested M13mp10-038/004 (inactive orientation), M13mp10-038/008 (active orientation), p BR322-038, and pCMS1, respectively. Lanes 7, 9, 12, and 16 contain the same unrestricted DNAs. The two cloning vectors used were included in lanes 4 and 5 (M13mp10) and in lanes 13 and 14 (pBR322) (restricted and unrestricted, respectively). Variously restricted lambda DNAs were used as a molecular size marker in lanes 3, 10, 17 and 18. Two empty lanes occur on either side of the gel (lanes 1, 2, 19, and 20). (B) Southern blot of the gel in panel A to which ^{32}P-labeled *opd* (nick-translated pBR322-038) was hybridized. The lanes correspond to those described for panel A.

native *Pseudomonas* background) resulted from utilizing the ampicillin gene promoter of the vector.

Insertion of the 1.3-kb *Pst*I fragment into the multiple cloning site of M13mp10 produced an *opd*-encoding phage (M13mp10-038/008) possessing an inducible (isopropyl-β-D-thiogalactopyranoside) whole-cell activity in *E. coli* JM103. Parathion was hydrolyzed by the phage-infected cells with a specific activity of approximately 10% of that of the native pseudomonad. This phage was used in hybridization studies ("C-tests") to select other isolates which possessed similarly sized insertions but lacked activity. In all cases, strains with hydrolytic activity gave negative C-tests with other active clones ("M13 Cloning/Dideoxy Sequencing Manual," BRL) (data not shown). Each of the negative isolates tested (M13mp10-038/003 and M13mp10-038/004) demonstrated positive C-test hybridization with the active clones, indicating that they contained the *opd* gene in the opposite orientation. These data were consistent with directional information provided by the pBR322 cloning.

Southern blotting with *opd* probe. Figure 1 summarizes the results of Southern hybridization of the *opd*-encoding replicons with ^{32}P-labeled, nick-translated pBR322-038 DNA (26). In each case, the clones which exhibited hydrolytic activity (or which had been previously shown to possess that sequence in the opposite orientation) hybridized to the probe (lanes 6 through 9, 11 and 12, and 15 and 16). Lanes 6, 8, and 11 of Fig. 1 demonstrate that each clone containing *opd* regenerated a 1.3-kb fragment which comigrated with the same sized fragment of the native plasmid (lane 15). These studies verify that the native plasmid encoded a plasmid-mediated, parathion-degrading activity on a 1.3-kb *Pst*I fragment.

Nucleotide sequencing. Dideoxy sequencing along both strands of the *opd* gene revealed a potential translational reading frame of 975 base pairs, and the DNA sequence verified the known restriction pattern for the *opd*-encoding fragment (Fig. 2). Five oligonucleotide primers were constructed for the purposes of sequencing regions lacking convenient restriction sites. In all cases, these primers efficiently promoted DNA synthesis.

The open reading frame (CTC-GGC-ACC) began 12 base pairs from the 5′ *Pst*I site and continued to a position at 1,038 base pairs before encountering a pair of closely spaced TGA stops (Fig. 3). A potential start site (ATG) was located 17 codons into the open reading frame. This codon appeared to be a candidate for the translational start since it is preceded by an AAGCAA sequence 15 base pairs upstream; the sequence and spacing are in good agreement with known *Pseudomonas* ribosomal binding sites (11, 15). In addition, several potential Rho-dependent terminator structures ranging in free energy of association from −12.6 to −15.4 kcal/mol (ca. −52.7 to −64.4 kJ/mol) were located 3′ of the open reading frame (data not shown).

This predicted amino acid sequence would give rise to a protein of 35,418 daltons before posttranslational modifications, if any. However, there are other potential start sites further into the sequence which would give rise to slightly smaller proteins (Fig. 3). In particular, valine 7 represents a possible start since GTG (formylmethionyl) codons are known in *Pseudomonas* spp. (8) and since a potential ribosomal binding site was located for this start site. However, the insertion of a *Bam*HI linker into the *Sph*I site (Fig. 2) disrupted the first putative ATG translational start site, and these genetic constructions possessed no enzymatic activity.

Amino acid sequencing of fusion polypeptides. When a fusion protein was constructed between the 5′ region of the *opd* gene and the *lacZ* gene at the *Aval-Sma*I site, a hybrid polypeptide was recovered, purified, and subjected to amino acid sequencing. Amino acid sequencing confirmed the

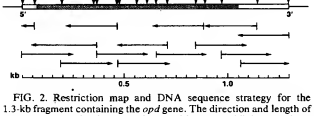

FIG. 2. Restriction map and DNA sequence strategy for the 1.3-kb fragment containing the *opd* gene. The direction and length of sequence determinations are shown with arrows (kilobase scale at bottom). The placement of the various restriction sites, exonuclease-generated subclones, and synthetic DNA primers used in the sequencing is shown along the fragment. The putative coding region for the *opd* gene is shaded.

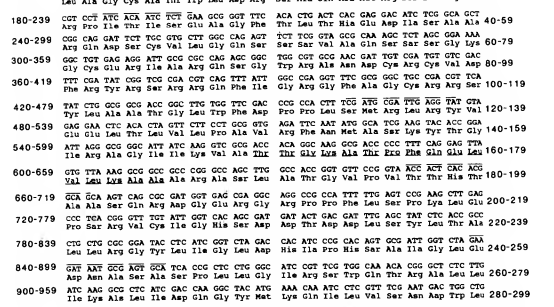

FIG. 3. Nucleotide sequence of the *opd* gene fragment. The amino acid sequence corresponding to the open reading frame beginning with the first ATG codon is identified below the sequence. Primers used in the sequencing are shown above the nucleotide sequence by overlining. The 3′ stop codon is indicated with a period. The amino acids confirmed by protein sequencing are underlined.

predicted reading frame for 16 amino acids 5′ of the fusion junction (Fig. 3). The sequence is 168 amino acids away from the presumed translational start for the *opd* gene product; however, truncated polypeptides are typical of fusions of membrane proteins with β-galactosidase (28), and proteolysis in the heterologous background may have produced a posttranslationally modified polypeptide.

Subcloning regional deletions. Figure 4 summarizes results obtained with various subclones of the 1.3-kb fragment containing the *opd* gene. Deletions outside the putative coding region remained active when the sequence was properly oriented for expression from the *lacZ* promoter. If the orientation was reversed or if deletions were made within the putative coding region, activity was eliminated.

DISCUSSION

The gene (*opd*) encoding a broad-substrate-range phosphotriesterase of *P. diminuta* MG has been shown to be encoded on a 50- to 60-kb plasmid (15, 23, 24; C. S. McDaniel, Ph.D. dissertation, Texas A&M University, College Station, 1985). The plasmid-borne gene was contained within a 1.3-kb restriction fragment and was transferred into a variety of plasmid and phage vectors and expressed in *E. coli*. The 1.3-kb fragment encoding the *opd* gene was sequenced, and its proper reading frame was confirmed by protein sequencing. The *opd* gene contained within the 1.3-kb fragment of the native plasmid possessed an open reading frame of 325 codons preceded by a 5′ flanking region with translational signals typical of other bacterial genes (8, 11, 16). In addition, the disruption of the presumed translational start site by the insertion of a *Bam*HI linker destroyed phosphotriesterase production. The predicted size of the deduced gene product and the size limitations defined by subcloned fragments are consistent with a predicted monomeric molecular weight of 35,418. Column chromatography of a detergent-treated cell extract demonstrated an enzymatic activity at 60,000 to 65,000 M_r which suggests that individual monomers might dimerize to form a holoenzyme. A similar phosphotriesterase has been described from *Flavobacterium* sp. strain ATCC 27551 (4), in which the hydrolytic activity was associated with a protein estimated to be greater than 50,000 M_r.

Restricted applications of chlorinated hydrocarbon pesticides, as the result of their inherent environmental hazards, have led to increased use of carbamate and organophosphorus pesticides. However, these applications have been compromised by the presence of soil bacteria capable of rapidly degrading the organosphosphorus compounds (21). The potential transfer of plasmid-mediated pesticide detoxification genes through a variety of hosts has important

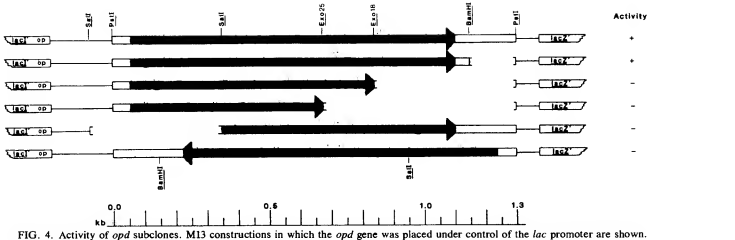

FIG. 4. Activity of *opd* subclones. M13 constructions in which the *opd* gene was placed under control of the *lac* promoter are shown. Sequences are adjusted to align vector DNA on either side of the *opd* subclone. Deletions are indicated by open space between brackets. The putative coding region for the *opd* gene is indicated by shading, and the sense direction is shown by arrows.

implications relative to the loss of efficacy of these biolabile pesticides. The potential mobility of these plasmid-borne genes may be analogous to the reduction of antibiotic efficacy in clinical and agricultural situations by plasmid-borne resistance factors (29).

It is clear that many soil bacteria possess degradative, plasmid-borne genes which could be readily transferred and expressed among a variety of bacterial and viral hosts. This phenomenon is not limited to organophosphorus neurotoxins, since plasmid-borne genes for degradative enzymes of herbicides have been well documented (9, 27). In the case of the *opd* genes, a wide range of pesticides sharing a common chemical structure are degraded (6), providing the potential for rapid evolution of genes to degrade a variety of pesticides and challenging the agrochemical rationale of substituting pesticides of similar chemical structure or increasing application rates for extended pest control. Rapid mutational adaptation in an enriched soil bacterial population could render ineffective any subsequent applications of a similar chemical.

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